Studies on Chitin IX. Specific Binding of Calcium Ions by Carboxymethyl-Chitin

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ABSTRACT: Carboxymethylation of chitin was carried out effectively for preparing a cation exchange resin under basic conditions. The resulting carboxymethyl-chitin (CM-chitin) was found to bind calcium ions specifically among alkali-earth metals even in the presence of monovalent cations such as sodium or potassium. Differential IR absorption spectroscopy suggested that the binding of calcium ion by carboxyl groups was assisted by acetamide groups at C_2 and primary hydroxyl groups at C_6 or secondary hydroxyl groups at C_3 in the N-acetylglucosamine residue. The binding constants were estimated at various ionic strengths and ion concentrations. The selectivity coefficient (K_{Na}^{Ca}) of CM-chitin for calcium ions over sodium ions was assumed to be 45.6 at neutral pH and 0.1—0.2 of ionic strength. The fibrous CM-chitin was prepared to investigate the effects of an increase in the number of surface ionic sites and degree of orientation on the binding capacity. The results indicated a marked enhancement.
    KEY WORDS   Carboxymethyl-Chitin / Specific Binding / Cation Exchange Resin / Differential IR Spectrum / Selectivity Coefficient / Fibrous CM-Chitin / Adsorption Chromatography /

Chitin, a natural abundant polysaccharide and a supporting material of crustacea, is reinforced by calcium carbonate in association with proteins in nature. But the binding property of chitin toward calcium ion is known to decrease upon purification. The affinity of chitin toward calcium ion is not abolished completely even after purification and, the specific binding ability toward calcium ion is expected to be reproduced when ionic binding sites become available on the chitin molecule by chemical modification. On this basis, carboxymethyl groups were introduced onto the chitin molecule by a reaction of alkali-chitin with monochloroacetic acid in isopropyl alcohol. The selective binding property toward calcium or magnesium ion also seems to be of interest in elucidating the ion pump mechanism of cell membranes, since the N-acetylglucosamine residue, a unit monomer of the chitin molecule, is one of the main components of a cell wall.

In this study, carboxymethylated chitin with a low degree of substitution and insoluble in water, was employed as an ion exchanger to simplify the reaction system. The specific adsorption of calcium ion was reproduced by the carboxymethylation, and this property persisted even in the presence of monovalent neutral salts such as KCl or NaCl. The highest degree of adsorption was observed toward Ca^{2+} among the divalent cations of alkali-earth metals. From the differential IR absorption spectrum between CM-chitin and calcium-bound CM-chitin, the binding was suggested to be a tetrahedral chelation supported by the carboxyl, acetamide and hydroxyl groups at C_3 or C_6 position of the N-acetylglucosamine residue. Magnesium ion bound to CM-chitin was released from the CM-chitin molecule in the presence of calcium ion owing to a low equilibrium constant. Such a selective adsorption property toward Ca^{2+} was also clearly indicated from the investigation on the Ca^{2+}–Mg^{2+} mixed system.

EXPERIMENTAL

Chitin

Chitin was prepared from Queen Crab shells according to the method of Hackman and pow-

dered to a 45—60 mesh before use.

Reagents

Reagents of reagent grade were purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. CMC was obtained from Seikagaku Kogyo Co., Ltd.

Alkali-Chitin

Chitin powder (20 g) was suspended in 80 ml of a 40% sodium hydroxide solution containing 0.2% of sodium dodecylsulfate (SDS) at 4°C. The slurry was made to stand at $-20°C$ overnight after aging for 1 h at 4°C.

Carboxymethylation of Chitin

Frozen alkali-chitin was suspended in 200 ml of isopropyl alcohol at room temperature and monochloroacetic acid was added stepwise for 4 h until the mixture was neutralized with mechanical stirring.[1,2] The product was collected by filtration and washed with ethanol and water thoroughly. Na-type carboxymethyl-chitin was obtained as an air dried powder after washing with acetone.

Methylation of Chitin

Chitin was methylated by the procedures reported previously.[3]

Degrees of Carboxymethylation and Methylation

The degree of carboxymethylation was estimated by potentiometric titration with 0.1 M HCl in 0.1 M NaCl under a nitrogen atmosphere at room temperature using Radiometer's Autotitrator TTT1c-SBR2c. CHN analysis was carried out to estimate the degree of substitution of CM- and methyl-chitins with Yanagimoto CHN Corder MT-2.

Infrared Absorption Spectra

IR and differential IR spectra were obtained on the KBr pellets using a JASCO A-302 IR spectrophotometer.

Adsorption Chromatography

11 g of CM-chitin powder (45—60 mesh) was loaded into a column (2.3 × 20.5 cm) after being damped well with water. The adsorption chromatography was carried out at a flow rate of 0.6 ml min^{-1} at room temperature after thorough washing with water or a 0.1 M KCl solution. The adsorption

of univalent cations was estimated by a pH shift of the effluent due to hydrogen chloride formed by the decomposition of the neutral salt. The adsorption of divalent cations on CM-chitin was measured by titration of the eluted ions with EDTA using the Eriochrome Black T (BT) or 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid (NN) of a Dotite indicator.[4] The calcium ion concentration in the mixture of magnesium and calcium ions was estimated by titration with an EDTA–NN system at pH 13 following removal of magnesium hydroxide by the addition of an 8 M KOH solution in the presence of traces of KCN and hydroxamine hydrochloride.[5] Strontium and barium ions were also titrated by EDTA, using Calcein Blue according to Elsheimer's method.[6]

The selectivity coefficient was obtained by the following equation,

$$K^M = \frac{N^{n+1}}{(E_t - N)^n (C - N)}$$

where E_t is the total ion exchange capacity (m eq), C (relative capacity), the volume of a cationic solution at the equilibrium point (m eq) and N, the amount of adsorbed cation (m eq) eluted by a 0.1 N HCl solution after being thorough washed with water. Though two kinds of equilibrium points were observed in the adsorption chromatography of calcium and magnesium ions, the last cations to be adsorbed disappeared as a result of a thorough washing with water or 0.1 M KCl: The exchange equilibrium between magnesium and calcium ions was measured by passing the calcium solution through the magnesium-bound CM-chitin layer at a flow rate of 0.6 ml $min.^{-1}$

Preparation of CM-Chitin Fiber

10 g of CM-chitin powder (DS = 0.28) was dissolved in 200 ml of 99% formic acid and the swollen material was frozen at $-20°C$ for 10 h. 30 ml of dichloroacetic acid (DCA) was added to the solution after thawing at room temperature. The spinning solution thus prepared was filtered through a flannel at a pressure of 1.0 kg/cm^{-2}, and spun into acetone as the 1st coagulation bath and a 50% aqueous methanol solution as the 2nd bath through a platinum nozzle (0.1 mmϕ, 50 holes) at room temperature. The fiber was stretched in air (stretched ratio, 1.4) and washed thoroughly with methanol to remove any traces of formic acid or

DCA. The air dried fiber was cut into pieces 1—2 mm in length. The fibrous CM-chitin was suspended in water and packed into a column (2.0 × 30.0 cm).

It was activated wtih 0.1 M NaOH after thorough washing with water, and washed with water again before applying metal ions to be adsorbed.

RESULTS AND DISCUSSION

Degree of Carboxymethylation

The degree of carboxymethylation was determined by both elemental analysis (DS: 0.25/N-acetylglucosamine residue) and potentiometric titration (0.28/residue) as shown in Table I along with the theoretical value. The extent of deacetylation during the carboxymethylation reaction was estimated to be less than 5% as shown by the potentiometric titration curve in Figure 1. The pK_a values of the carboxyl and amino groups were assumed to be 3.4 and 6.4, respectively, from the titration curve.

The presence of carboxyl groups was also indicated by an absorption peak at $1730 \, cm^{-1}$ in the IR spectrum in Figure 2, which appeared after treating the Na-type CM-chitin with 0.1 M HCl.

Adsorption Chromatography

CM-chitin was found to adsorb calcium ions, and there was a big difference in the adsorption capacity in the H-type and Na-type CM-chitins. Since the amount of adsorbed calcium ion is identical with a half mole of the carboxyl group on the chitin, the ionic binding seems to be supported by the electrostatic force between calcium ion and two carboxylate ions in the CM-chitin molecule. Chitin was also found to adsorb Ca^{2+} weakly as shown in Figure 3, although this adsorbed ion was washed out competely from the chitin by a thorough wash-

ing with water. The trapping site in the chitin molecule seems to consist of the primary hydroxyl group at C_6 and acetamide group at C_2 of the N-acetylglucosamine residue, since there was little trapped Ca^{2+} in the 1.2 methylated chitin, in which initial alkylation site was assumed to be the primary hydroxyl group at C_6 as reported previously.[7] The adsorption curves of CM-chitin for divalent cations showed at least two equilibrium points independent
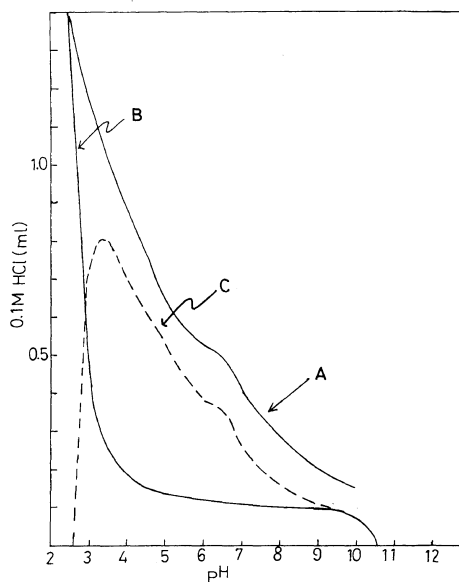

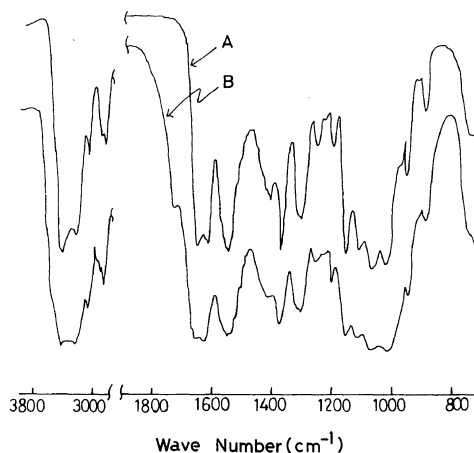**Figure 1.** Titration curve of CM-chitin in 0.1 M NaCl at room temperature: (A), CM-chitin; (B), Blank; (C), (A)—(B).

Figure 2. IR spectra of chitin (A) and CM-chitin (B).

Table I. Elemental analysis for CM-chitin

	Found	Calcd for 0.25 CM-chitin[a]
	%	%
C	44.3	45.2
H	6.5	6.5
N	6.4	6.4

[a] Calculated on the assumption that the chitin molecule contains $1/2 H_2O$ per N-acetylglucosamine residue.
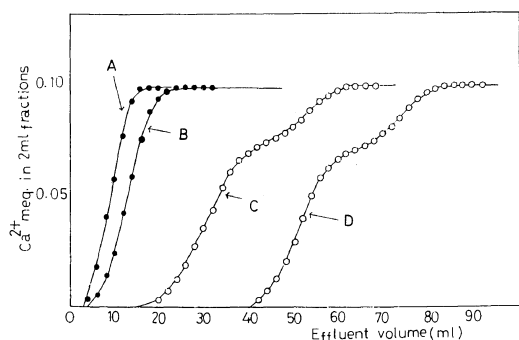
Figure 3. Adsorption chromatography of 0.05 M CaCl₂ on chitin and CM-chitin: (A), chitin (H-type); (B), chitin (Na-type); (C), CM-chitin (H-type); (D), CM-chitin (Na-type).

Table II. Adsorption capacities and selectivity coefficients of chitin, chitin derivatives and CM-cellulose (CMC)

Resin	Type	Solutions	Capacity[a] m equiv. g⁻¹	K[b]
Chitin	H	0.1 M NaOH	0.24	—
	H	0.05 M MgCl₂	0.13	1.30
	H	0.05 M CaCl₂	0.15	3.40
	Na	0.05 M CaCl₂	0.16	4.40
CM-chitin	H	0.1 M NaOH	0.61	—
	H	0.1 M NaCl	0.00	0.00
	H	0.1 M KCl	0.00	0.00
	H	0.05 M MgCl₂	0.26	0.98
	H	0.05 M CaCl₂	0.39	6.89
	H	0.05 M SrCl₂	0.16	0.44
	H	0.05 M BaCl₂	0.20	0.62
	Na	0.05 M MgCl₂	0.13	0.91
	Na	0.05 M CaCl₂	0.50	45.60
Methyl-chitin[c]	H	0.05 M MgCl₂	0.00	0.00
	H	0.05 M CaCl₂	0.00	0.00
CM-cellulose	H	0.1 M NaOH	1.00	—
	H	0.05 M MgCl₂	0.10	trace
	H	0.05 M CaCl₂	0.17	0.03
	Na	0.05 M CaCl₂	0.57	2.63

[a] Capacity (m eq g⁻¹) including that of chelated ions and that of trapped washable ions.
[b] Selectivity coefficient.
[c] 1.2 Methylated chitin.

of the type of activation. It was found that the last Ca²⁺ to be adsorbed on CM-chitin could be washed out with water as in the case of that adsorbed on chitin, and the amount of trapped washable Ca²⁺ was approximately the same as that trapped on chitin. The net capacity for the Na-type CM-chitin was thus estimated to be 0.08 m eq g⁻¹. The adsorption capacities and selectivity coefficients of chitin, chitin derivatives and CM-cellulose (CMC) are summarized in Table II. The Na-type CM-chitin adsorbs Ca²⁺ 6.6 times more strongly than the H-type (K_{Na}=45.60, K_H=6.89), and there is a remarkable difference in the selectivity coefficients of Ca²⁺ and Mg²⁺ or other alkali-earth metal ions as shown in Table II. The strikingly high affinity of CM-chitin toward Ca²⁺ seems to suggest the formation of a chelate complex between CM-chitin and the calcium ion because of the large difference in selectivity constants of CM-chitin and CMC. The selectivity constant of CM-chitin toward Ca²⁺ is 230 times higher than that of CMC[8] (H-type) and 17 times higher than that of CMC (Na-type) as shown in Table II (H-type: CM-chitin, K=6.89; CMC, K=0.03. Na-type; CM-chitin, K=45.60; CMC, K=2.63).

As shown in Figure 4, the optimum ionic strength of Ca²⁺ adsorption toward CM-chitin was found to be 0.13—0.20 and the highest degree of adsorption was obtained at a calcium ion concentration of 10—20 mM at room temperature. Though this capacity became remarkably small by lowering the Ca²⁺ concentration, the ionic strength seemed a more important factor than the Ca²⁺ concentration.
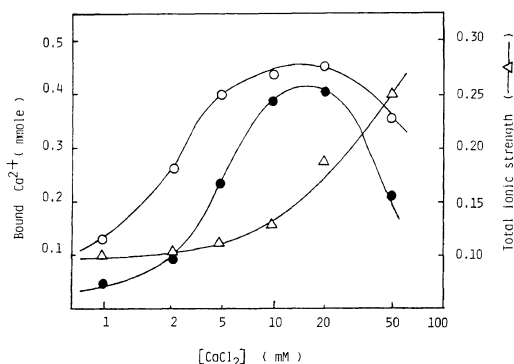

Figure 4. Dependency of Ca²⁺ adsorption capacity on Ca²⁺ concentration and the ionic strength. In each case 0.1 M KCl or 0.1 M NaCl was added. —○—, K-type; —●—, Na-type; —△—, total ionic strength.

Therefore, a larger adsorption capacity may be possible with a higher ionic strength at a low
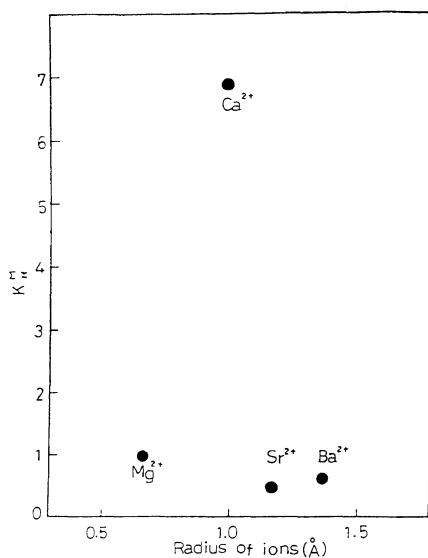
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Figure 5. Relationship between the selectivity coefficient of CM-chitin (H-type) and the radius of alkali-earth metal ions.

Table III. Adsorption capacities and distribution coefficients (K^{Ca}/K^{Mg}) of CM-chitin

Type	Ions[a]	Capacity (m eq g^{-1})			Distribution[b] coefficient
		Ca	Mg	Total	
–COOH	$Ca^{2+} + Mg^{2+}$	0.08	0.02	0.10	4.00
–COOK	$Ca^{2+} + Mg^{2+}$	0.11	0.06	0.17	1.83
–COONa	$Ca^{2+} + Mg^{2+}$	0.19	0.04	0.23	4.75

[a] Ionic strength of the solution is 0.23 (0.05 M $CaCl_2 + 0.05$ M $MgCl_2 + 0.1$ M NaCl or KCl).
[b] Ca^{2+}/Mg^{2+}.

calcium concentration.

The selectivity coefficients of the CM-chitin (H-type) with alkali-earth metals are summarized in Figure 5 as a function of the radius of the ion. The selectivity constant for the calcium ion is almost 7 times higher that of other alkali-earth metals as shown in the figure even on the H-type CM-chitin. The ratio between the selectivity constants of Ca^{2+} and Mg^{2+} becomes 50 when CM-chitin is activated with NaOH as shown in Table II ($K_{Na}^{Ca}/K_{Na}^{Mg} = 45.6/0.91 = 50.1$). In the exchange chromatography between Mg^{2+} and Ca^{2+}, 0.035 m eq g^{-1} of Mg^{2+} was unwashable by a 0.05 M $MgCl_2$ solution applied to
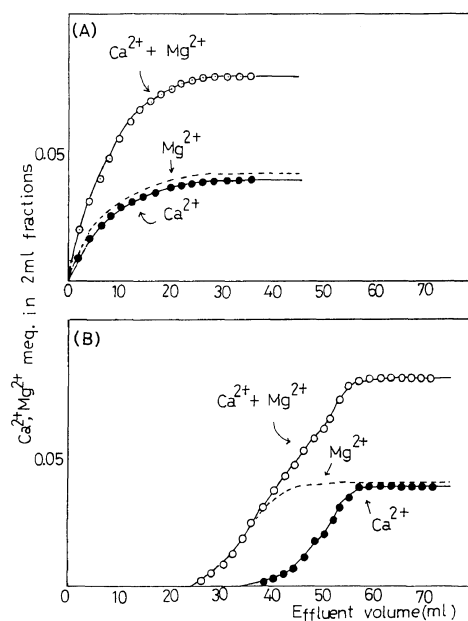

Figure 6. Adsorption chromatographies of the mixed solutions of 0.05 M $MgCl_2$ and 0.05 M $CaCl_2$ on CM-cellulose (A) and CM-chitin (B) in the presence of 0.1 M KCl or NaCl.

the Na-type CM-chitin, and 0.029 m eq g^{-1} of Mg^{2+} was released from the above Mg-type CM-chitin gradually by washing with a 0.05 M $CaCl_2$ solution (82.9% recovery). A mixed solution of 0.05 M $MgCl_2$ and 0.05 M $CaCl_2$ was applied to the three different types of CM-chitin to confirm this observation. The adsorption capacities and distribution coefficients (K^{Ca}/K^{Mg}) are listed in Table III. The highest value was obtained by the Na-type CM-chitin and the lowest one by the K-type CM-chitin in the presence of 0.1 M NaCl or KCl. CMC showed no selectivity between Ca^{2+} and Mg^{2+} as evident from Figure 6A. On the other hand, CM-chitin showed a high selectivity toward calcium ion as shown in Figure 6B.

The amount of unwashable Ca^{2+} on the fibrous CM-chitin increased to 0.30 m eq g^{-1} from 0.19 m eq g^{-1} of the powdered one. The enhancement of this capacity may have resulted from the increase in surface area or the orientation of the molecules. The X-ray diffraction pattern of the calcium-bound CM-chitin fiber suggests an increase in the regularity of arrangement of the carboxyl groups compared to that of the powdered Ca-bound CM-chitin.
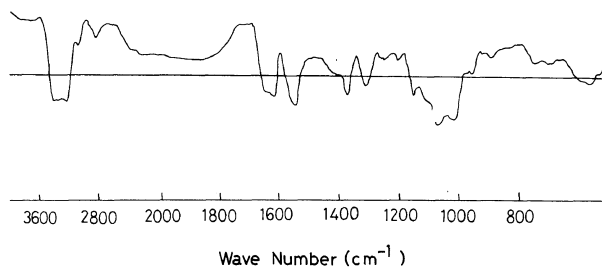
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Wave Number (cm^{-1})

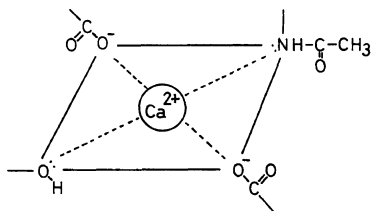**Figure 7.** Differential IR absorption spectrum between Ca^{2+}-bound CM-chitin and CM-chitin.

Figure 8. Schematic drawing for the binding of calcium ion on CM-chitin.

The differential IR absorption spectrum between the CM-chitin and Ca^{2+}-loaded CM-chitin is shown in Figure 7 for the Na-type CM-chitin. The absorptions due to amide A (3350 cm^{-1}), amide I (1660 cm^{-1}), amide II (1555 cm^{-1}) and hydroxyl groups (1050 and 1110 cm^{-1}) changed remarkably by the adsorption of Ca^{2+} ion and so apparently the tetrahedral chelation of CM-chitin toward Ca^{2+} is assisted by the acetamide and hydroxyl groups in addition to carboxyl groups. A plausible scheme for the calcium chelation on CM-chitin is shown in Figure 8. Identification of the chelation site is now under investigation using NMR with a water soluble CM-chitin, since formic acid, the only solvent for the water insoluble CM-chitin, tends to release the bound calcium ion from CM-chitin.

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